

# Fluoroaluminates activate transducin-GDP by mimicking the $\gamma$ -phosphate of GTP in its binding site

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Fluoride activation of the cGMP cascade of vision requires the presence of aluminum, and is shown to be mediated by the binding of one  $\text{AlF}_4^-$  to the GDP/GTP-binding subunit of transducin. The presence of GDP in the site is required:  $\text{AlF}_4^-$  is ineffective when the site is empty or when  $\text{GDP}\beta\text{S}$  is substituted for GDP. This sensitivity to the sulfur of  $\text{GDP}\beta\text{S}$  suggests that  $\text{AlF}_4^-$  is in contact with the GDP. Striking structural similarities between  $\text{AlF}_4^-$  and  $\text{PO}_4^{3-}$  lead us to propose that  $\text{AlF}_4^-$  mimics the role of the  $\gamma$ -phosphate of GTP.

Fluoride    Aluminum    Phosphate    Transducin    G-protein    Adenylate cyclase

## 1. INTRODUCTION

Fluorides have long been known to influence the activity of hormone-sensitive adenylate cyclase systems [1] and of the analogous cGMP phosphodiesterase (PDE) system of retinal rod outer segments (ROS) [2]. The target of  $\text{F}^-$  is the  $\alpha$ -subunit of the GTP-binding proteins, called G/F,  $\text{N}_s$ ,  $\text{N}_i$  or  $\text{G}_s$ ,  $\text{G}_i$  in hormonal systems, and transducin (T) in the retina [3,4]. These proteins activate the cyclase, or PDE, when loaded with GTP by a GTP/GDP exchange catalysed by liganded hormone receptor, or light-activated rhodopsin. They become inactive when the GTP is hydrolysed to GDP. In a normal activation-deactivation cycle [5], with nucleotides in the medium, the site on  $\text{T}_\alpha$  is virtually always occupied: it is occluded, and the nucleotide is non-exchangeable until  $\text{T}_\alpha$  binds to  $\text{R}^*$  [6]. This binding causes the site to open and allows a fast exchange of the bound GDP for GTP; then  $\text{T}_\alpha \cdot \text{GTP}$  quickly dissociates from  $\text{T}_{\beta\gamma}$  and from  $\text{R}^*$ , with the GTP

again occluded in the site where it will eventually be hydrolysed to GDP.  $\text{T}_\alpha \cdot \text{GTP}$  leaves the membrane and activates PDE by binding to its inhibitor subunit. The nucleotide site may be emptied by removing all guanyl nucleotides in the presence of  $\text{R}^*$ . In this case  $\text{T}_\alpha \cdot \text{T}_{\beta\gamma}$  will not dissociate from  $\text{R}^*$  until its decay to opsin.

Fluorides in the millimolar range confer upon the GDP-bound protein structural and functional properties of the GTP-bound one [4,8]. The requirement of a trace amount of aluminum was recently demonstrated for fluoride activation of hormonal systems and it was suggested that  $\text{AlF}_4^-$  was indeed the active species [9]. We demonstrate that in the retinal system one  $\text{AlF}_4^-$  activates with high affinity one  $\text{T}_\alpha \cdot \text{GDP}$  molecule. The presence of GDP is required:  $\text{AlF}_4^-$  has no effects on  $\text{T}_\alpha$  when the nucleotide site is empty or contains  $\text{GDP}\beta\text{S}$ , even though  $\text{T}_\alpha \cdot \text{GDP}\beta\text{S}$  is functionally and structurally similar to  $\text{T}_\alpha \cdot \text{GDP}$ . The sensitivity of  $\text{AlF}_4^-$  to the sulfur of  $\text{GDP}\beta\text{S}$  suggests that  $\text{AlF}_4^-$  binds close to the GDP. Striking structural similarities between  $\text{AlF}_4^-$  and  $\text{PO}_4^{3-}$  lead us to propose that  $\text{AlF}_4^-$  interacts with the GDP in the nucleotide site, where it mimics the role of the  $\gamma$ -phosphate of GTP.

**Abbreviations:**  $\text{GDP}\beta\text{S}$ , guanosine 5'-O-(3-thiodiphosphate);  $\text{R}^*$ , photoexcited rhodopsin

## 2. MATERIALS AND METHODS

These studies were conducted with purified bovine ROS membranes [10], in which the native content of transducin ( $\sim 1/10$  rhodopsins) and PDE was preserved, or alternatively with reconstituted systems: purified transducin subunits, in various states [11], and partially purified PDE were added back to ROS membranes that had been washed free of all peripheral proteins and in which rhodopsin was virtually the only protein component left.  $T_{\alpha}\cdot\text{GDP}$  (or permanently activated  $T_{\alpha}\cdot\text{GTP}\gamma\text{S}$ ) and  $T_{\beta\gamma}$  were purified separately or as an associated  $T_{\alpha}\cdot\text{GDP}\cdot T_{\beta\gamma}$  complex [11]. In native or reconstituted systems, functionality was assessed by monitoring cGMP hydrolysis by the pH-metric method [12]. As characterized by Kühn [7,10], inactive  $T_{\alpha}\cdot\text{GDP}$  and active  $T_{\alpha}\cdot\text{GTP}$  (or  $T_{\alpha}\cdot\text{GTP}\gamma\text{S}$ ) were also differentiated by their binding affinities:  $T_{\alpha}\cdot\text{GDP}$  binds strongly to  $T_{\beta\gamma}$  and the complex binds to ROS membranes in the dark at moderate ( $\sim 10^{-1}$  M) ionic strength and binds strongly to  $R^*$  upon illumination; in contrast,  $T_{\alpha}\cdot\text{GTP}\gamma\text{S}$  dissociates from  $T_{\beta\gamma}$  and does not bind to ROS membranes in the dark or to  $R^*$ . Activation of the system by GTP or  $\text{GTP}\gamma\text{S}$  is therefore always correlated with separation of  $T_{\alpha}$  from the membrane and its solubilization in moderate ionic strength media [7,10]. Structural differences between  $T_{\alpha}\cdot\text{GDP}$  and  $T_{\alpha}\cdot\text{GTP}\gamma\text{S}$  were also observed through their different sensitivity to proteolytic enzymes [13].

## 3. RESULTS AND DISCUSSION

In the presence of NaF and  $\text{AlCl}_3$ , it was first observed that purified  $T_{\alpha}\cdot\text{GDP}$  acquired the capacity to activate PDE in reconstituted systems in the dark with an efficiency comparable to that of  $T_{\alpha}\cdot\text{GTP}\gamma\text{S}$ . Under the same conditions, in dark native membranes,  $T_{\alpha}\cdot\text{GDP}$  dissociates quantitatively from  $T_{\beta\gamma}$  and the membrane. The requirement for aluminum can only be demonstrated when working in clean plastic vessels: as pointed out in [9], common glassware releases sufficient amounts of aluminum to elicit activation in the presence of NaF. The quantitative requirements for  $\text{AlCl}_3$  and NaF are shown in fig.1. Maximum efficiency is achieved at an NaF concentration

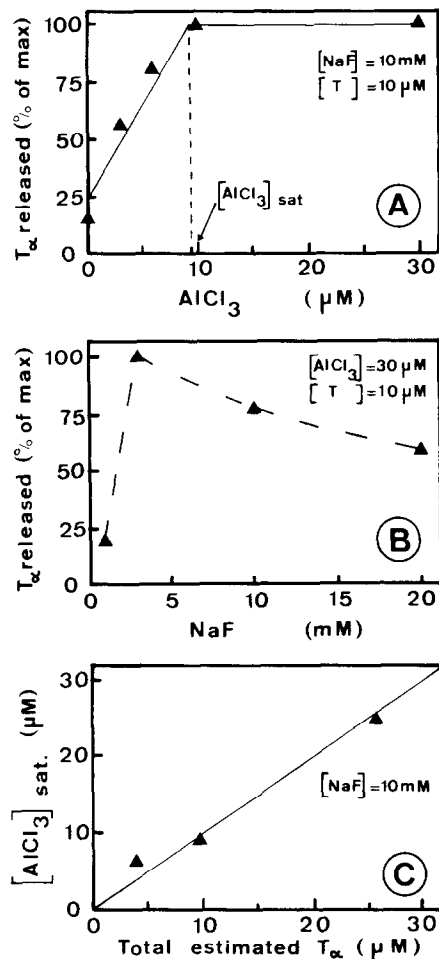


Fig.1. Demonstration of the activation of  $T_{\alpha}$  by a stoichiometric amount of  $\text{AlF}_4^-$ . The activation of  $T_{\alpha}$  was monitored through its solubilization from dark ROS membranes in moderate ionic strength medium [7,10]. Buffer composition (in mM): Hepes, 5 (pH 7.5); NaCl, 120;  $\text{MgCl}_2$ , 0.5; DTT, 1. After washing once in this medium, the ROS membrane pellet was resuspended, and NaF and  $\text{AlCl}_3$  were added sequentially. The suspension was incubated for 6 min at  $20^\circ\text{C}$  and centrifuged. Total transducin concentration was estimated as  $1/10$  that of rhodopsin.  $T_{\alpha}$  extracted in the supernatant was determined by densitometry on Coomassie blue-stained SDS-PAGE (10% acrylamide [19]). Similar results were obtained by the assay of Bradford [20] of the proteins in the supernatant. (A) Requirement for  $\text{AlCl}_3$  at 10 mM NaF. (B) Requirement for NaF at 30  $\mu\text{M}$   $\text{AlCl}_3$ . (C) Concentration of  $\text{AlCl}_3$  required to saturate the release of  $T_{\alpha}$  (as shown in A for 10  $\mu\text{M}$   $T_{\alpha}$ ), at various total transducin concentrations. Assuming that all of the aluminum is complexed into  $\text{AlF}_4^-$  [14,15], the straight line fit demonstrates a stoichiometry of one  $\text{AlF}_4^-$  per  $T_{\alpha}$ .

where the predominant fluoroaluminate complex ion is  $\text{AlF}_4^-$  [14,15]. Dose-activation curves were run at various  $T_\alpha$  concentrations. Full activation was always obtained with total  $\text{AlF}_4^-$  concentrations corresponding to one  $\text{AlF}_4^-$  per  $T_\alpha$  (fig.1C). This demonstrates a stoichiometry of one  $\text{AlF}_4^-$  bound per  $T_\alpha$  and a binding constant of the order of  $1 \mu\text{M}$  or higher.  $\text{AlF}_4^-$ -activated  $T_\alpha \cdot \text{GDP}$  has the proteolytic sensitivity characteristics of  $T_\alpha \cdot \text{GTP}\gamma\text{S}$  [4].

As observed by Stein et al. [4] illumination appeared to cancel the effects of  $\text{AlF}_4^-$  on PDE activation as well as on the binding properties of  $T_\alpha$ . In contrast to the suggestion of Stein et al., this is not due to the blocking of access to the 'fluoride site' by the previous binding of  $R^*$  to  $T_\alpha$ : when  $\text{AlF}_4^-$  is applied first in the dark, solubilization of  $T_\alpha$  and activation of PDE are reversed upon strong illumination;  $T_\alpha$  appears to bind quantitatively to  $R^*$  in the presence of  $\text{AlF}_4^-$  as it does in the absence of  $\text{AlF}_4^-$ . The presence of  $T_{\beta\gamma}$  is required for this rebinding to take place, as it is for the binding of  $T_\alpha \cdot \text{GDP}$  to  $R^*$  [7]. We noticed, however, that the deactivation by strong illumination of  $\text{AlF}_4^-$ -activated  $T_\alpha$  is a slow process, which appears to require a few minutes to develop. A significant observation was then that a few micromolar GDP, which does not interfere with the binding of  $T_\alpha$  to  $R^*$  in the absence of  $\text{AlF}_4^-$ , or activate the system in the absence of  $\text{AlF}_4^-$ , suffices in the presence of  $\text{AlF}_4^-$  to prevent or reverse the rebinding of  $T_\alpha$  to  $R^*$  and to preserve or restore the PDE activation (fig.2). Our interpretation is that  $\text{AlF}_4^-$ -activated  $T_\alpha \cdot \text{GDP}$  (as does probably  $T_\alpha \cdot \text{GTP}$ ) still has a low affinity for  $T_{\beta\gamma}$  and  $R^*$ . A very small proportion of  $\text{AlF}_4^-$ -activated  $T_\alpha \cdot \text{GDP}$  binds back to  $T_{\beta\gamma}$  and to  $R^*$ , then the site opens, the bound GDP is released and, in the absence of nucleotide in the medium it is not replaced, as the affinity of the site is in the  $10^{-5}$  M range [6]. With no nucleotide in the site,  $T_\alpha$  cannot be activated by  $\text{AlF}_4^-$ , the binding of  $T_\alpha$  to  $R^*$  becomes irreversible, and progressively all of the  $T_\alpha$  becomes bound. A few micromolar GDP in the medium suffices to keep a GDP in the open site of an  $R^*$ -bound  $T_\alpha$  and to prevent this phenomenon.  $\text{AlF}_4^-$  alone, without nucleotide in the site, is therefore unable to activate  $T_\alpha$ : binding of a GDP molecule and of an  $\text{AlF}_4^-$  is concurrently required. Indications that the  $\text{AlF}_4^-$ -binding site is in close proximity to the

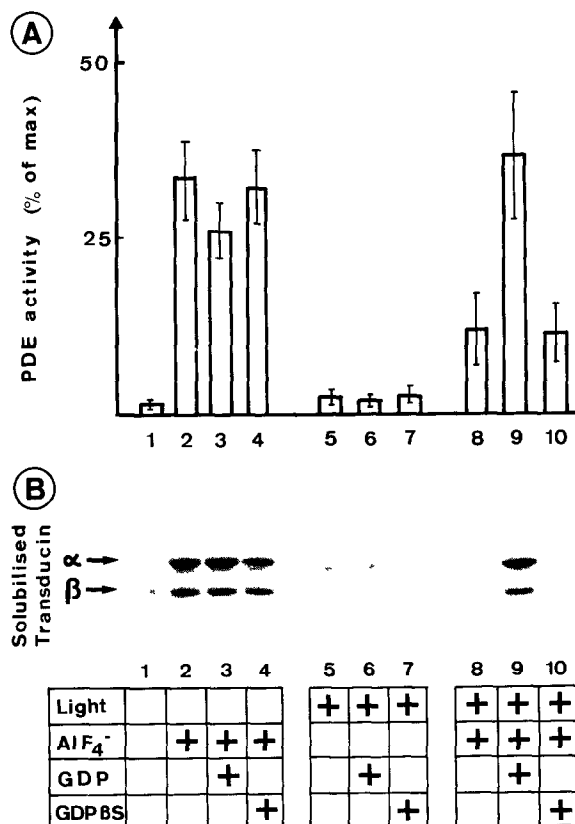


Fig.2. Demonstration of the requirement for the presence of GDP in the site for  $\text{AlF}_4^-$  activation. Measurements are performed on aliquots of the same ROS membrane preparation. Incubation conditions are shown in the grid below. Light: flash photoexciting ~90% of the rhodopsin.  $\text{AlF}_4^-$ :  $30 \mu\text{M}$   $\text{AlCl}_3^-$  +  $10 \text{ mM}$   $\text{NaF}$ . GDP and GDP $\beta\text{S}$ :  $100 \mu\text{M}$ . (A) PDE activity measured by the pH-metric method [12], normalized to the maximal activity observed upon illumination in the presence of GTP $\gamma\text{S}$ . Buffer composition (in mM): Hepes, 10 (pH 7.5); KCl, 120;  $\text{MgCl}_2$ , 2; cGMP, 0.4. ROS membrane concentration:  $12 \mu\text{M}$  rhodopsin. (B) Solubilization of  $T_\alpha$ , observed on SDS-PAGE (same procedure as in fig.1). In the dark (lanes 1–4),  $T_\alpha$  has retained its native GDP occluded in the site [6].  $\text{AlF}_4^-$  alone activates the system; added GDP and GDP $\beta\text{S}$  have no effects. Upon illumination in the absence of  $\text{AlF}_4^-$  (lanes 5–7),  $T_\alpha$  binds to  $R^*$ , and added GDP and GDP $\beta\text{S}$  do not activate the system. Upon illumination and in the presence of  $\text{AlF}_4^-$  (lanes 8–10), in the absence of added GDP (lane 8),  $T_\alpha$  remains bound to  $R^*$  and inactive. The nucleotide site is open, its dissociation constant being  $\sim 20 \mu\text{M}$  [6], most of the GDP has been released, and under this condition,  $\text{AlF}_4^-$ -induced activation is quenched (cf. lanes 8 and 2). Addition of GDP restores  $\text{AlF}_4^-$ -induced activation (lane 9) but GDP $\beta\text{S}$  is ineffective.

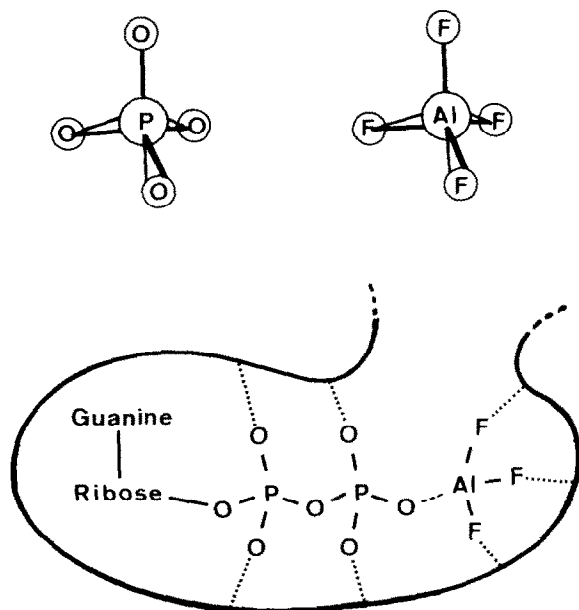


Fig.3. Structural analogies between  $\text{PO}_4^{3-}$  and  $\text{AlF}_4^-$  and schematic model proposed for the interaction of  $\text{AlF}_4^-$  with the GDP occluded in its site in  $\text{T}_\alpha$ .

nucleotide-binding site are provided by the use of GDP analogs.  $\text{GDP}\beta\text{S}$  can be substituted for GDP by washing out the GDP of  $\text{R}^*$ -bound  $\text{T}_\alpha$ , adding  $\text{GDP}\beta\text{S}$  and letting  $\text{R}^*$  decay to opsin.  $\text{T}_\alpha \cdot \text{GDP}\beta\text{S}$  seems to retain all of the characteristics of  $\text{T}_\alpha \cdot \text{GDP}$ : it binds to  $\text{T}_{\beta\gamma}$  and to  $\text{R}^*$ , and exchanges its nucleotide for GTP in the presence of  $\text{R}^*$ , like  $\text{T}_\alpha \cdot \text{GDP}$  (T.M. Vuong and M. Chabre, unpublished).  $\text{T}_\alpha \cdot \text{GDP}\beta\text{S}$  also has the proteolytic sensitivity characteristics of  $\text{T}_\alpha \cdot \text{GDP}$  (not shown). However,  $\text{T}_\alpha \cdot \text{GDP}\beta\text{S}$  is not activated by  $\text{AlF}_4^-$ , as if the binding of the ion complex were directly hindered by the presence of the large sulfur atom on the  $\beta$ -phosphate. The observation that fluorides were inactive in the presence of  $\text{GDP}\beta\text{S}$  had previously been done on the hormone sensitive G-protein systems where, by contrast, fluoride activation was effective when the analog  $\text{GP-CH}_2\text{-P}$  had been substituted for GDP [16]. Our results and these previous findings strongly suggest that the fluoroaluminate complex binds in the nucleotide site, close to the  $\beta$ -phosphate of GDP.

It then struck us that the structure of the complex ion  $\text{AlF}_4^-$  is very similar to that of a phosphate group  $\text{PO}_4^{3-}$  [17,18]: both ions are tetrahedral, the

central aluminum atom has the same size as a phosphorus atom; fluoride has a Van der Waals radius of 1.35 Å, like oxygen, and the Al-F bond length is 1.65–1.70 Å vs 1.55–1.60 Å for the average P-O bond length in  $\text{PO}_4^{3-}$ . We therefore propose that the activation of transducin (and of the analogous  $\text{G}_s$  and  $\text{G}_i$  proteins) by fluorides results from the binding of an  $\text{AlF}_4^-$  into the guanine nucleotide site, close to the  $\beta$ -phosphate of GDP (fig.3).  $\text{F}^-$  is known to be able to make hydrogen bonds which could substitute for that made by the oxygens of the phosphate group. The bound  $\text{AlF}_4^-$  simulates the presence of the bound  $\gamma$ -phosphate of GTP and therefore confers on the protein the structure of the active  $\text{T}_\alpha \cdot \text{GTP}$  state. The binding of  $\text{AlF}_4^-$  to GDP and the protein site must be tighter than that of a phosphate group, which is released from the protein after the hydrolysis of the  $\gamma$  P-O-P bond of GTP. This release is spontaneous, and we have checked that phosphate ions are not activators of the system, even at millimolar concentrations. The high electronegativity of  $\text{F}^-$  may account for this tight binding.

This model could also account for the effect of fluorides, or more likely, aluminofluoride complexes in other enzymatic systems involving phosphate groups, such as phosphatases.

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